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The Lymphotoxin-β Receptor Induces Different Patterns of Gene Expression via Two NF-κB Pathways

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Summary

The lymphotoxin-β receptor (LTβR) plays critical roles in inflammation and lymphoid organogenesis through activation of NF-κB. In addition to activation of the classical NF-κB, ligation of this receptor induces the processing of the cytosolic NF-κB2/p100 precursor to yield the mature p52 subunit, followed by translocation of p52 to the nucleus. This activation of NF-κB2 requires NIK and IKKα, while NEMO/IKKγ is dispensable for p100 processing. IKKβ-dependent activation of canonical NF-κB is required for the expression but not processing of p100 and for the expression of proinflammatory molecules including VCAM-1, MIP-1β, and MIP-2 in response to LTβR ligation. In contrast, IKKα controls the induction by LTβR ligation of chemokines and cytokines involved in lymphoid organogenesis, including SLC, BLC, ELC, SDF1, and BAFF.

Introduction

The lymphotoxin (LT)-β receptor, a member of the TNF receptor superfamily, controls processes of differentiation crucial for the development and organization of lymphoid tissue (Locksley et al., 2001; Shakhov and Nedospasov, 2001). Mice deficient in the LTβR gene or genes encoding one of its ligands, LTα or LTβ, exhibit a complex phenotype characterized by a failure to form lymph nodes and Peyers's patches and the absence of natural killer (NK) and NK-T cells (reviewed in Ansel and Cyster, 2001; Fu and Chaplin, 1999). In the adult, LTβR signaling is required for maintaining the splenic architecture, the integrity of the marginal zone, and compartmentalization of T and B cells. During antigen-dependent responses, germinal centers and networks of follicular dendritic cell networks fail to form in LTβR-deficient mice. Chemokines such as secondary lymphoid tissue chemokine (SLC/CCL21) and Epstein-Barr virus-induced molecule 1 ligand (ELC/CCL19) play crucial roles in secondary lymphoid development and may require LTβR signaling for their expression in development (Ngo et al., 1999; Luther et al., 2000; Vassileva et al., 1999).

The NF-κB family of transcription factors regulates expression of genes crucial to innate and adaptive immune responses, cell growth, and apoptosis (Barkett and Gilmore, 1999; Silverman and Maniatis, 2001). In mammals, this family consists of five members that form homo and heterodimeric complexes including NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA (p65), RelB, and Rel (c-Rel). NF-κB activity is tightly controlled by a collection of inhibitory proteins that belong to the IκB family, which include IκBα, IκBβ, IκBϵ, Bcl-3, p100, and p105 (Siebenlist et al., 1994).

In most cells, the NF-κB dimer p50:RelA is sequestered in the cytosol and its nuclear translocation can be induced by a wide variety of stimuli. These stimuli activate the IκB kinase (IKK) complex, which contains two catalytic subunits, IKKα and IKKβ, and the regulatory subunit IKKγ (also called NEMO) (DiDonato et al., 1997; Regnier et al., 1997; Rothwarf and Karin, 1999; Rothwarf et al., 1998; Woronicz et al., 1997; Yamaoka et al., 1998; Zandi et al., 1997), responsible for phosphorylation of IκBα, IκBβ, or IκBϵ (Karin and Ben-Neriah, 2000). The disruption of genes encoding individual IKK subunits have demonstrated that IKKβ and IKKγ transmit the response to most common NF-κB-inducing agents, whereas IKKα has an unexpected role in keratinocyte differentiation (Hu et al., 1999, 2001; Li et al., 1999a, 1999b, 1999c; Makris et al., 2000; Rudolph et al., 2000; Takeda et al., 1999; Tanaka et al., 1999; Yamaoka et al., 1998). IKKα kinase activity is essential for induction of IκB degradation in mammary epithelial cells in response to RANK ligand (Cao et al., 2001) and in the basal processing of the NF-κB2/p100 precursor to p52 in B lymphocytes (Senftleben et al., 2001). Processing of p100 is negatively regulated by a processing-inhibitory domain (PID) and positively regulated by the NF-κB-inducing kinase (NIK) (Xiao et al., 2001). Using an overexpression system, Xiao and coworkers initially proposed that NIK is the direct upstream kinase involved in p100 processing. However, another study has shown that NIK-induced p100 processing in B cells depends on IKKα (Senftleben et al., 2001). The physiological regulators of this pathway have not been described.

Results

The Control of Gene Expression following Ligation of LTβR

We assessed the effect of LTβR ligation on the expression of several genes with roles in inflammation and immune development using an agonistic, LTβR-specific monoclonal antibody (see Experimental Procedures). In mouse embryonic fibroblasts (MEF), this antibody induced the expression of the adhesion molecule VCAM-1, one of the known target genes downstream of LTβR (Matsumoto et al., 1999), induced by NF-κB (Shu et al., 1993) (Figure 1A). This effect was not seen in MEFs lacking LTβR.

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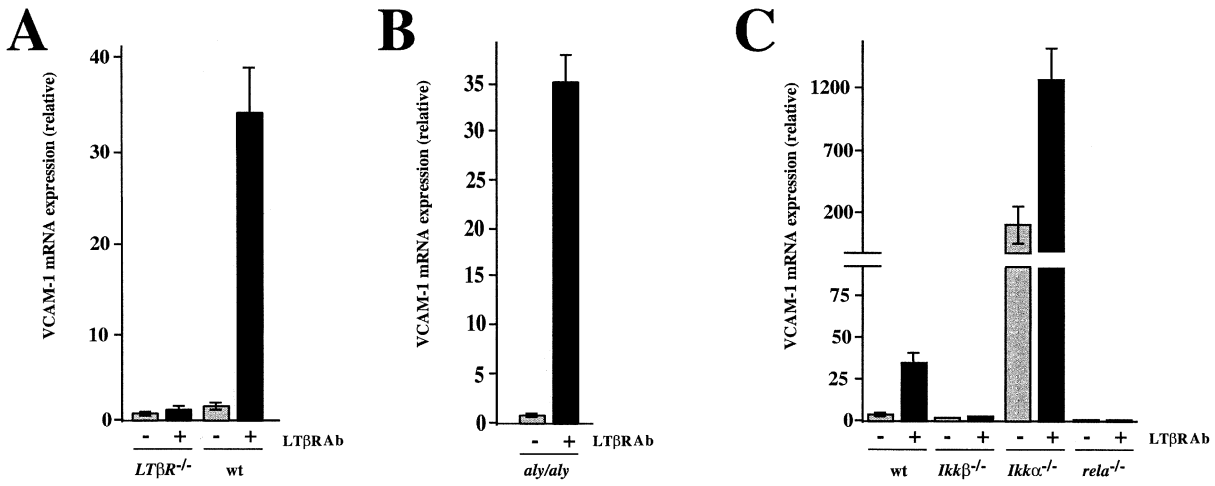

Figure 1. LTβR-Induced VCAM-1 Expression Depends on IKKβ and RelA but Not on NIK or IKKα

(A) Ligation of LTβR induces VCAM-1 mRNA upregulation. Wild-type and LTβR-deficient MEFs were treated (+) or not (-) for 3 hr with 2 μg/ml of agonistic anti-LTβR antibody prior to RNA extraction. VCAM-1 mRNA expression level was monitored by real-time PCR. Absolute VCAM-1 mRNA values were determined and reported relative to the basal expression level detected in untreated LTβR$^{-/-}$ MEF.

(B) VCAM-1 mRNA expression is induced in *aly/aly* MEFS upon LTβR ligation. Cells were treated as analyzed as described in (A).

(C) LTβR-mediated VCAM-1 mRNA upregulation requires IKKβ and RelA. Wild-type, *Ikk*β$^{-/-}$, *Ikk*α$^{-/-}$, and *rela*$^{-/-}$ MEFs were treated as in (A). VCAM-1 mRNA expression level was monitored by real-time PCR. Absolute VCAM-1 mRNA values were determined and reported relative to the basal expression level detected in untreated wt MEF.

Recently it was suggested that VCAM-1 expression is dependent on the function of NIK as cells from NIK mutant *aly/aly* mice (Shinkura et al., 1999) did not upregulate this protein upon LTβR ligation (Matsumoto et al., 1999). We therefore examined the expression of VCAM-1 mRNA by real-time PCR in *aly/aly* MEFs. Ligation of LTβR induced increased expression of VCAM-1 mRNA (Figure 1B). In addition, anti-LTβR induced IκB-kinase activity in both wt and *aly/aly* MEF (data not shown). Therefore, the *aly/aly* mutation does not inhibit the canonical NF-κB activation pathway that leads to expression of this gene. The lack of upregulation of VCAM-1 by FACS in *aly/aly* cells observed by others (Matsumoto et al., 1999), which we have also observed (data not shown), may represent posttransciptional regulation of VCAM-1.

We therefore examined the expression of VCAM-1 mRNA by real-time PCR in MEFs deficient for proteins involved in NF-κB activation. Ligation of LTβR in wt MEFs induced a pronounced increase in expression of VCAM-1 mRNA, which was not seen in cells lacking IKKβ or RelA (Figure 1C). In contrast, constitutive expression of VCAM-1 mRNA was elevated in cells lacking IKKα and this was substantially increased by ligation of LTβR. Therefore, induction of NF-κB (p50:RelA) by anti-LTβR is responsible for the upregulation of VCAM-1 expression. Our results show that transcriptional induction of VCAM-1 requires neither IKKα nor NIK but does require both IKKβ and RelA.

We then examined the effect of an intraperitoneal injection of the agonistic anti-LTβR antibody on expression of several genes that are likely to be involved in inflammation, lymphoid organ development, and homeostasis. The anti-LTβR caused a significant upregulation in the spleens of wt mice of the mRNA for the chemokines macrophage inflammatory protein-1β (MIP-1β/CCL4), macrophage inflammatory protein-2 (MIP-2), secondary lymphoid tissue chemokine (SLC/CCL 21), EBI-1-ligand chemokine (ELC/CCL 19) B lymphocyte chemoattractant (BLC/CXCL 13), and stromal cell-derived factor-1 α (SDF-1-α/CXCL 12) (Figures 2A and 2B). We also found that the B cell activation factor BAFF (BlyS/TALL-1/THANK) (Moore et al., 1999) was upregulated by ligation of LTβR.

Recently, a knockin mouse strain in which two serines within the activation loop of IKKα are replaced by alanines (*Ikk*α^{AA}) was generated (Cao et al., 2001). Unlike *Ikk*α$^{-/-}$ animals, *Ikk*α^{AA} mice are viable but show defects in basal processing of p100 to the p52 subunit of NF-κB2, the maturation of B lymphocytes, and the development of lymphoid organs (Senftleben et al., 2001). In the *Ikk*α^{AA} knockin mice, the anti-LTβR antibody failed to stimulate the expression of SLC, ELC, BLC, SDF-1-α, or BAFF mRNA (Figure 2B). In contrast, the mutation in the activation loop of IKKα enhanced the upregulation of MIP-1β and MIP-2 mRNAs following ligation of LTβR (Figure 2A). An increase in NF-κB activity in these knockin mice has been described (Senftleben et al., 2001). Further, we observed that NFκB2/p100 mRNA was upregulated by ligation of LTβR in the spleens of both wild-type and knockin *Ikk*α^{AA} mice (Figure 2C).

Ligation of the LTβR Stimulates NIK- and IKKα-Dependent p100 Processing

The processing of p100 to p52 is an important step in the activation of NF-κB2. Therefore, wt and mutant MEFs were treated with anti-LTβR and the processing of p100 was examined by immunoblotting. The low level of constitutive processing of p100 to p52 was dramatically enhanced by ligation of LTβR in wild-type (wt) but not *LTβR*$^{-/-}$ MEFs (Figure 3A). Recently, cells from NIK mutant *aly/aly* mice were shown to lack spontaneous

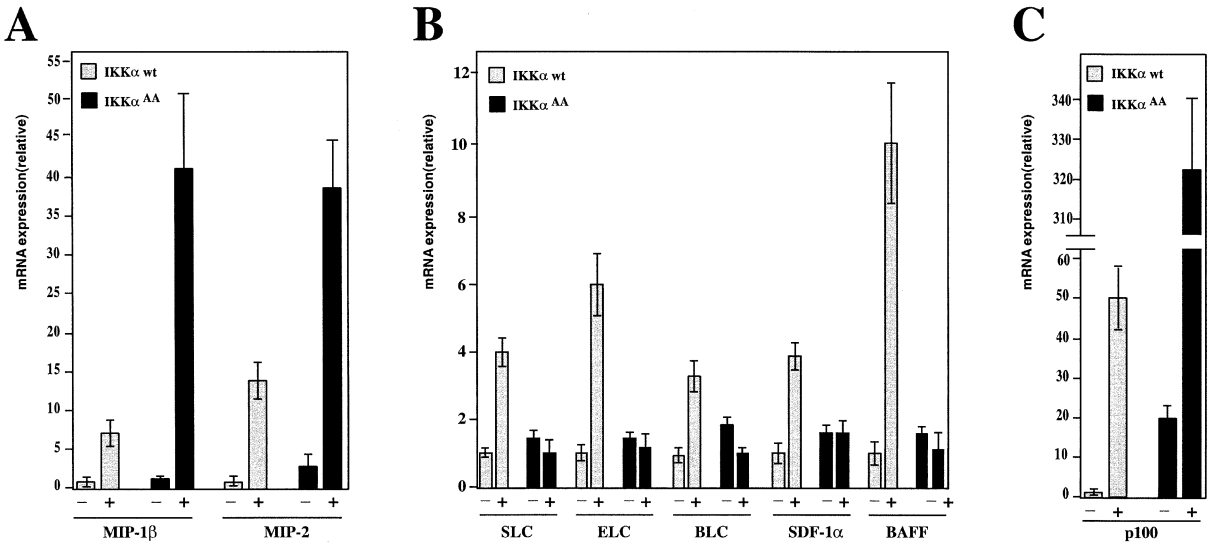

Figure 2. Expression of Chemokines and Cytokine in Wild-Type and *Ikk*α^{AA} Knockin Splenocytes following Intraperitoneal Injection of Agonistic Anti-LTβR Antibody

Wild-type (n = 3) and *Ikk*α^{AA} knockin (n = 3) mice were injected intraperitoneally (as described in Experimental Procedures) with an isotype control antibody (-) or with an agonistic LTβR antibody (+). RNAs were isolated from spleens, reverse-transcribed, and analyzed by real-time PCR for (A) inflammatory chemokines MIP-1β and MIP-2; (B) for chemokines SLC, ELC, and the cytokines SDF-1α and BAFF; and (C) for NF-κB2/p100. For each gene, absolute mRNA values were determined and reported relatively to the basal expression (-) detected in wt IKKα mice. One representative experiment out of three is shown.

processing of p100 to p52 (Xiao et al., 2001), and ligation of the LTβR on *aly/aly* MEFs failed to induce the processing of p100 to p52 (Figure 3A). Therefore, the *aly* mutation appears to affect both basal and LTβR-induced processing of p100.

This effect of ligation of LTβR was not restricted to MEF. In the human colon carcinoma cell line HT-29, ligation of LTβR with an agonistic antibody for human LTβR or with the ligand LTαβ2 also resulted in the induction of processing of p100 to p52 (Figure 3B). This was inhibited by the proteasome inhibitor MG-132 (Xiao et al., 2001) but not by the caspase inhibitor zVAD-fluoromethylketone.

Cytokine-induced NF-κB activation is mediated by a high molecular weight IKK complex composed of IKKα, IKKβ, and NEMO/IKKγ. Using cells genetically deficient in these components (Hu et al., 1999; Li et al., 1999c, 2000; Makris et al., 2000), we examined their respective roles in the induction of processing of p100 to p52 following ligation of LTβR. Anti-LTβR stimulated the processing of p100 to p52 in wt MEFs but not in MEFs lacking IKKα (Figure 3C). Examination of the processing of p100 in wt MEFs suggests that new synthesis of p100 contributes to the increased accumulation of p52, and this was not seen in the cells lacking IKKβ. Nevertheless, in MEFs lacking IKKβ, the p100 was almost completely processed by 24 hr after ligation of LTβR. This pattern is consistent with NF-κB2 as a NF-κB target gene (Liptay et al., 1994), as the activation of NF-κB depends on IKKβ. Indeed, ligation of LTβR in MEFs lacking RelA (*rela*$^{-/-}$) failed to induce the accumulation of NF-κB2 although the processing of p100 remained intact (Figure 3D). This was the same pattern of p100 processing as was seen in MEFs lacking IKKβ (Figure 3C).

B cells from mice with a knockin mutation of IKKα (IKKα^{AA} mice) show a reduced constitutive processing of p100 (Senftleben et al., 2001). *Ikk*$\alpha^{-/-}$ MEFs were therefore reconstituted with retroviral expression vectors encoding wt IKKα or the IKKα mutant harboring the point mutations S176A and S180A in the activation loop (IKKα^{AA}) (Delhase et al., 1999) and stable lines were selected for GFP expression. In each case the stably transduced lines expressed similar amounts of IKKα protein (Figure 3E) and cell-surface LTβR by FACS (data not shown). *Ikk*$\alpha^{-/-}$ cells showed neither constitutive nor LTβR-induced p100 processing, while reconstitution with wt IKKα restored this activity (Figure 3E). However, the IKKα^{AA} mutant did not, indicating that activation of IKKα by phosphorylation within the T loop is required for the LTβR-induced of p100 processing.

Further, MEF lacking wt IKKα showed an increased level of p100 expression 24 hr after LTβR ligation (Figures 3C and 3E), consistent with the observations on p100 expression after in vivo administration of anti-LTβR (Figure 2C). Thus, while IKKβ and RelA participate in the regulation of the level of p100 available for processing to p52, IKKβ is dispensable for the induction of p100 processing in response to ligation of LTβR. IKKα, however, is required for the induction of this processing event.

LTβR Ligation Induces Nuclear Translocation of RelB-p52 and RelA-p52

As previously reported (Dejardin et al., 1998; Dobrzanski et al., 1995), RelB is mainly sequestered by the inhibitor p100, but the physiological signals that control RelB expression and nuclear translocation are still poorly characterized. We therefore examined the nuclear trans-

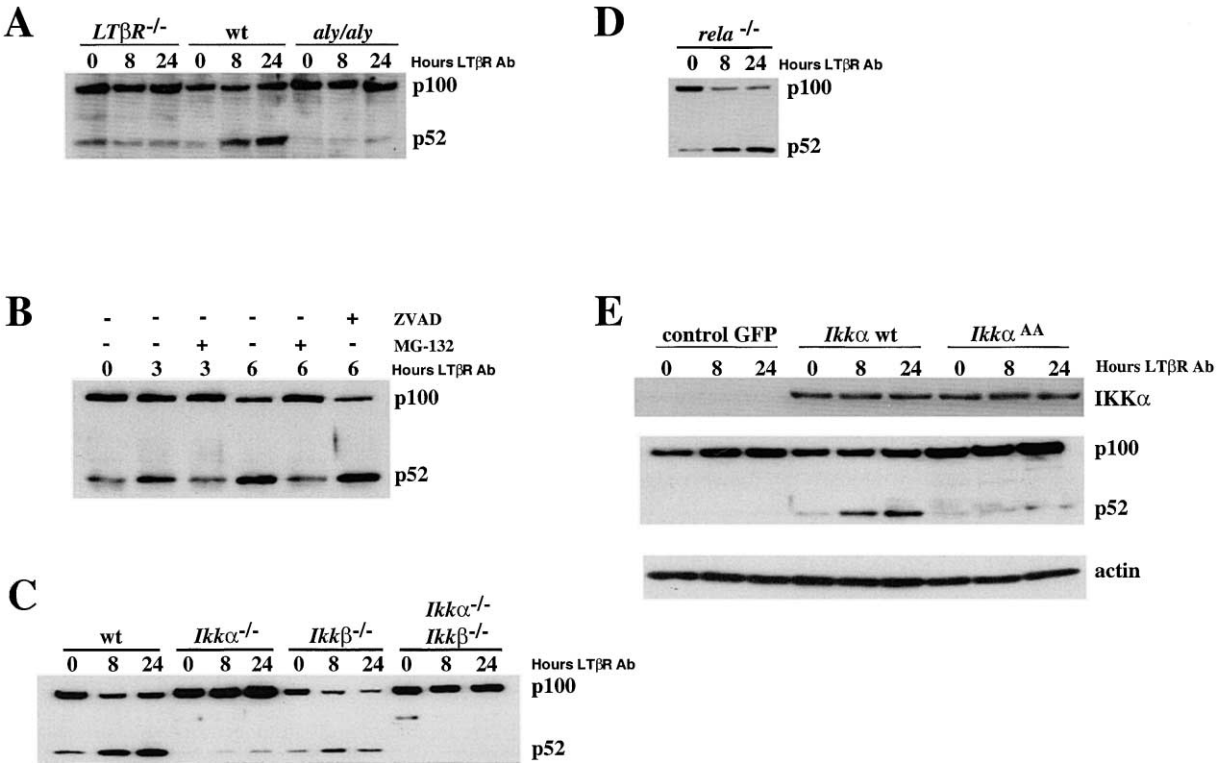

Figure 3. NIK and IKKα Are Required for the Inducible p100 Processing through Activation of the LTβR

(A) NIK is involved in LTβR-mediated p100 processing. Embryonic fibroblasts from *LTβR*$^{-/-}$, wild-type, or *aly/aly* mice were treated with an agonistic LTβR antibody (2 μg/ml) for 0, 8, or 24 hr. Whole cell extracts were separated by SDS-PAGE and p100/p52 proteins were analyzed by immunoblotting using an amino-terminal p52:p100-specific antibody.

(B) p100 processing is inhibited by the proteasome inhibitor MG-132 but not by the caspase inhibitor ZVAD. HT-29 cells were treated with a human LTβR agonistic antibody (1 μg/ml) for 0, 3, or 6 hr in the presence or absence of MG-132 or zVAD-fmk. Whole cell extracts were analyzed as in (A).

(C) IKKα but not IKKβ is required to trigger p100 processing. Embryonic fibroblasts from wild-type, *Ikk*α$^{-/-}$, *Ikk*β$^{-/-}$, or *Ikk*α$^{-/-}$ *Ikk*β$^{-/-}$ mice were treated and analyzed as described in (A).

(D) RelA is involved in the sustained production of p100 following LTβR ligation. Embryonic fibroblasts from *rela*$^{-/-}$ mice were treated and analyzed as described in (A).

(E) LTβR-induced p100 processing requires activation of IKKα. Embryonic fibroblasts from IKKα-deficient mice were infected with either an IRES-GFP control retrovirus or an IRES-GFP retrovirus expressing either wt IKKα or the mutant IKKα^{AA}. Stably infected cells were sorted by FACS to >95% GFP-expressing cells. Cells were treated with agonistic anti-LTβR (2 μg/ml) and cell extracts were subjected to immunoblotting.

location of RelB and p52 following ligation of the LTβR. The levels of RelB and p52 in the nucleus increased within 1–2 hr after LTβR ligation and continued to accumulate over 24 hr (Figure 4A). In addition, the overall level of RelB expression increased following stimulation with anti- LTβR. In contrast, RelA translocated to the nucleus within 1–2 hr of anti-LTβR treatment and then decreased, while in *LTβR*$^{-/-}$ MEFs RelA and RelB remained in the cytosol (data not shown). The same patterns of nuclear translocation of NF-κB subunits were also seen upon LTβR ligation in HT29 cells (data not shown). The upregulation and translocation of RelB in response to LTβR-ligation occurred in *Ikk*β$^{-/-}$ MEFs, but not in MEFs lacking IKKα (Figure 4B).

The DNA binding activity of the nuclear NF-κB complexes was analyzed by electromobility shift assay (Figure 5A). Nuclear extracts from untreated HT29 cells contained only low levels of NF-κB DNA binding activity, which increased after 1 or 6 hr of treatment with agonistic anti-LTβR antibody, and this binding was effectively competed with unlabeled oligonucleotide. Treatment of the 1 hr extracts with antibody to RelA reduced (supershifted) the band indicated by the upper (filled) arrow in Figure 5A; this was not seen with antibody to RelB, and only slightly with antibody to p52. In contrast, at 6 hr this band was effectively supershifted with antibodies to p52, RelA, or RelB.

To determine whether RelA or RelB associate with p52, cytosolic and nuclear extracts from HT29 were precipitated with anti-RelA or -RelB antibodies and immunoblotted for p52 (Figure 5B). After 1 hr ligation of LTβR, p52 was found in association with RelA in the nucleus; after 6 hr ligation p52 was found in association with RelB, consistent with the patterns of nuclear translocation of each of these proteins (Figure 5C). Conversely, immunoprecipitation of p52 from nuclear extracts coprecipitated with RelA and RelB at the appropriate time points (data not shown). Therefore, while we suspect that RelA is associated predominantly with p50 after ligation of LTβR as described by others (Force et al., 2000), the p52 that translocates to the nucleus following LTβR ligation appears to associate with both RelA and RelB.

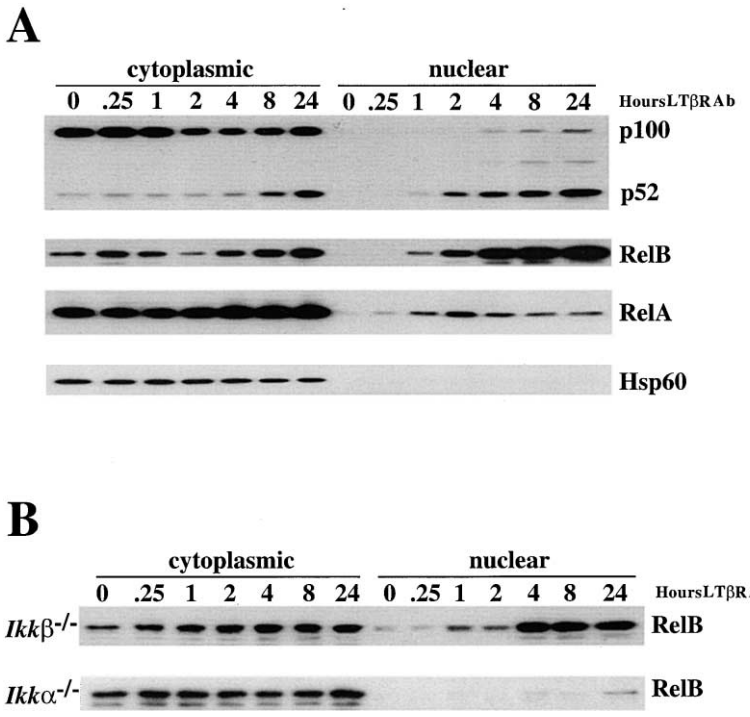

Figure 4. Nuclear Translocation of p52 and RelB Is Dependent on IKKα but Not on IKKβ

(A) Ligation of LTβR triggers p52, RelB, and RelA nuclear translocation. Mouse embryonic fibroblasts were treated with an agonistic LTβR antibody for the indicated period of time. Cytoplasmic and nuclear extracts were prepared as previously described (Dejardin et al., 1995) and analyzed by immunoblotting for p100:p52, RelB, and RelA proteins. As control for cytoplasmic contamination in the nuclear fractions, we used an anti-Hsp60 antibody.

(B) LTβR ligation-induced RelB nuclear translocation and RelB protein level upregulation are IKKα dependent but IKKβ independent. Embryonic fibroblasts from *Ikk*$\beta^{-/-}$ and *Ikk*$\alpha^{-/-}$ mice were treated as described in (A) and analyzed for RelB protein expression in both cytoplasmic and nuclear fractions.

Interestingly, nuclear p100 (also seen in Figure 4) coprecipitated with RelB but not with RelA (Figure 5B). This is consistent with a recent report that p100 may function in nuclear export of RelB (Solan et al. 2002).

C-Terminal Phosphorylation of p100 following Ligation of LTβR by an IKKα-Associated Kinase Activity

In most cultured cell types, IKKα and IKKβ are predominantly found in equimolar ratios within a high molecular weight IKK complex that also contains IKKγ (Rothwarf et al., 1998). To determine if ligation of LTβR activates IκB and p100 kinase activities, cytoplasmic extracts from MEFs stimulated for 0, 15, or 180 min with agonistic anti-LTβR antibody or with TNF were prepared. Extracts were immunoprecipitated with anti-IKKα and phosphorylation of IκBα and p100 were assessed. As shown in Figure 6A, both TNF and anti-LTβR triggered IKK kinase-mediated IκBα phosphorylation. In contrast, the ligation of LTβR but not the TNFR induced IKKα-associated p100 kinase activity (Figure 6B).

Treatment of IKKγ-deficient MEFs with agonistic anti-LTβR antibody also led to p100 kinase activity (Figure 6C). Interestingly, the activation of the IKKα-associated p100 kinase activity and p100 processing were elevated in cells lacking IKKγ. Moreover, despite the absence of IKKγ, ligation of LTβR further increased p100 processing. In each case, the activation of p100 kinase activity preceded the appearance of processed p100.

To gain insights into the possible complex responsible for p100 phosphorylation, 293T cells were transfected with expression constructs for flag-NIK, HA-IKKα, and/or HA-p100. Precipitation of NIK with anti-flag coprecipitated IKKα and p100, as expected from other studies (Matsushima et al., 2001; Xiao et al., 2001) (Figure 6D). To determine if all three proteins can form a complex, the precipitated NIK was eluted with flag peptide and the released proteins were then bound to anti-IKKα. The p100 coprecipitated with IKKα under these conditions (Figure 6D), indicating that it was in a complex with both NIK and IKKα. While we do not know if this complex forms physiologically, it raises the possibility that a NIK-IKKα-p100 complex is involved in the activation of NF-κB2 independently of IKKγ.

Discussion

Here we have studied the signaling events mediated by ligation of the LTβR that lead to the expression of genes involved in inflammation and secondary lymphoid organogenesis and homeostasis (Figure 7). We have observed that the induction of these genes occurs via the activation of two NF-κB pathways: the formation of p50:RelA heterodimers and the processing of the NF-κB2 gene product p100 to active p52.

The conversion of p100 to p52 has been shown to be negatively controlled by a C-terminal processing-inhibitory domain of p100 and activated by phosphorylation, with subsequent ubiqutination and degradation by the proteasome (Xiao et al., 2001). In that study, overexpression of either LTβR and/or NIK in HEK 293T cells resulted in p100 processing. Our observation that LTβR ligation leads to p100 processing in wt but not NIK mutant *aly/aly* MEFs is consistent with these results. Subsequent studies have demonstrated that NIK-induced p100 processing is IKKα dependent (Senftleben et al., 2001). Here we have shown that LTβR-induced p100 phosphorylation and processing are dependent on IKKα but independent of IKKβ. In addition, we have now shown that IKKγ is dispensable for p100 processing. The

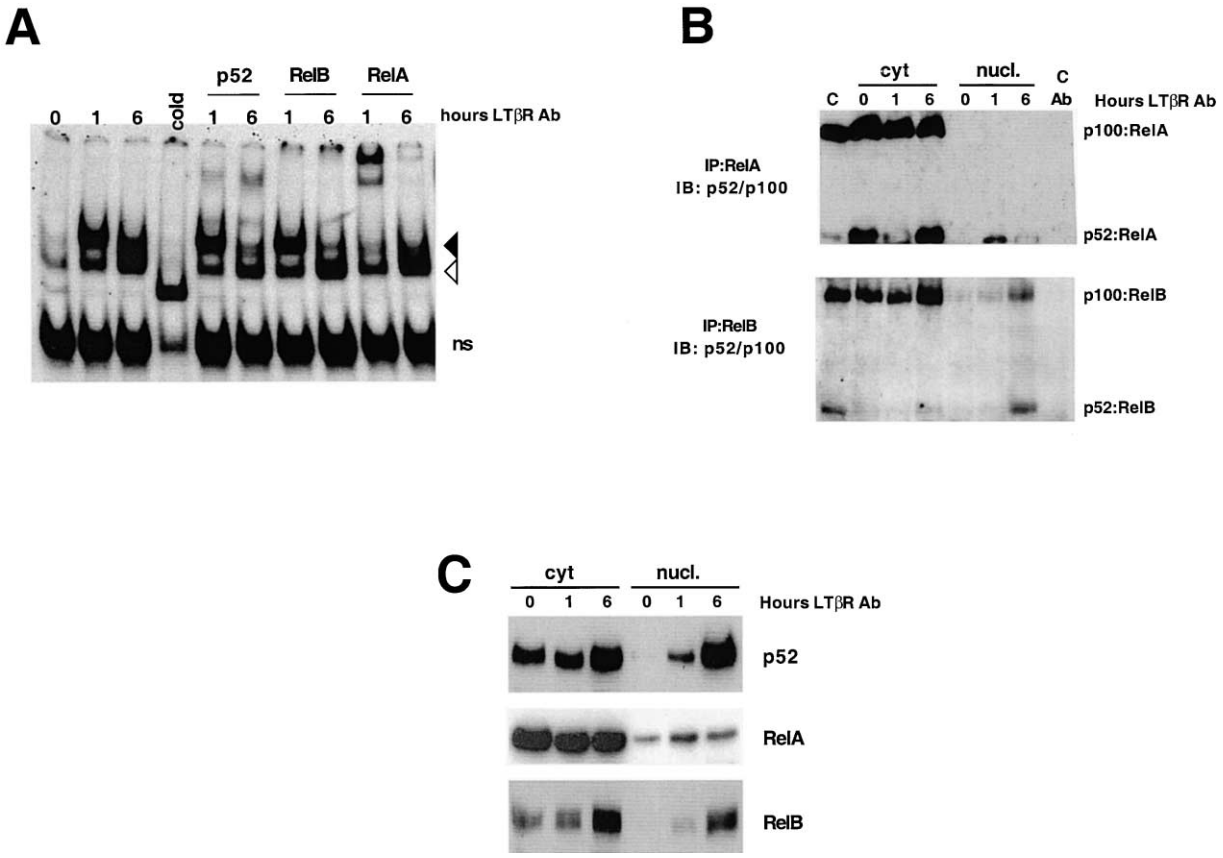

Figure 5. LTβR Ligation Induces Distinct p52-Containing Complexes

(A) DNA binding activity of NF-κB complexes following LTβR ligation. HT29 cells were treated with an agonistic anti-human LTβR antibody (10 μg/ml) for the indicated times and nuclear extracts were prepared and analyzed by electromobility shift assay for NF-κB binding activity. For supershift, samples were incubated with the indicated antibody prior to incubation with the labeled probe. Arrowheads show the NF-κB complexes.

(B) Identification of p52:RelA and p52:RelB complexes. HT29 cells were treated with agonistic anti-human LTβR antibody as in (A). Cytosolic (cyt.) and nuclear (nucl.) extracts were subjected to immunoprecipitation with anti-RelA or anti-RelB antibody and analyzed for associated p52/p100 by immunoblot. C, control extract; C Ab, immunoprecipitation of control cytostolic extract with control antibody.

(C) Nuclear translocation of p52, RelA, and RelB after LTβR ligation The cytosolic and nuclear extracts from the experiment shown in (B) were immunoblotted for the indicated proteins.

elevated p100 processing we observed in the absence of IKKγ may be a consequence of an increase in IKKα kinase activity as proposed previously (May et al., 2000). Although the absence of IKKγ/NEMO or the disruption of the interaction between IKKγ/NEMO and IKKs prevent TNF-induced IκB kinase activity (Makris et al., 2000; May et al., 2000), ligation of LTβR nevertheless induces p100 phosphorylation in the absence of IKKγ/NEMO. This provocative result suggests that IKKγ/NEMO is not a universal integrator for all NF-κB signaling pathways and it is possible that other IKK complexes exist.

Animals lacking LTα, LTβ, LTβR, or NIK have severe defects in secondary lymphoid organogenesis (Alimzhanov et al., 1997; Banks et al., 1995; De Togni et al., 1994; Futterer et al., 1998; Koni et al., 1997; Yin et al., 2001). This phenotype includes an absence of lymph nodes, Peyer's patches, germinal centers, and a disorganized splenic architecture. In the adult, LTβR signaling is required for maintaining the architecture of the white pulp in the spleen and is necessary for integrity of the marginal zone and segregation of T and B cells into discrete compartments. Although T and B cells develop normally and populate the spleen, during antigen-dependent responses germinal centers do not form and networks of follicular dendritic cells fail to form in LTβR-deficient mice (Futterer et al., 1998).

Similarly, animals lacking p100/p52 show a marked reduction in the B cell compartments of the spleen, bone marrow, and lymph nodes, and display an altered splenic and lymph node architecture, lacking B cell follicles and germinal centers (Caamano et al., 1998; Franzoso et al., 1998). Mice deleted for RelB are also unable to form germinal centers upon antigen challenge and display abnormal splenic architecture (Burkly et al., 1995; Weih et al., 1995). In addition, animals deficient in IKKα have defects in secondary lymphoid tissue, especially in Peyer's patch development, germinal center formation, and B cell development (Kaisho et al., 2001; Matsushima et al., 2001; Senftleben et al., 2001).

Cytokine-induced NF-κB activation through IκBα degradation does not require IKKα kinase activity and instead is dependent on IKKβ (Delhase et al., 1999; Hu et

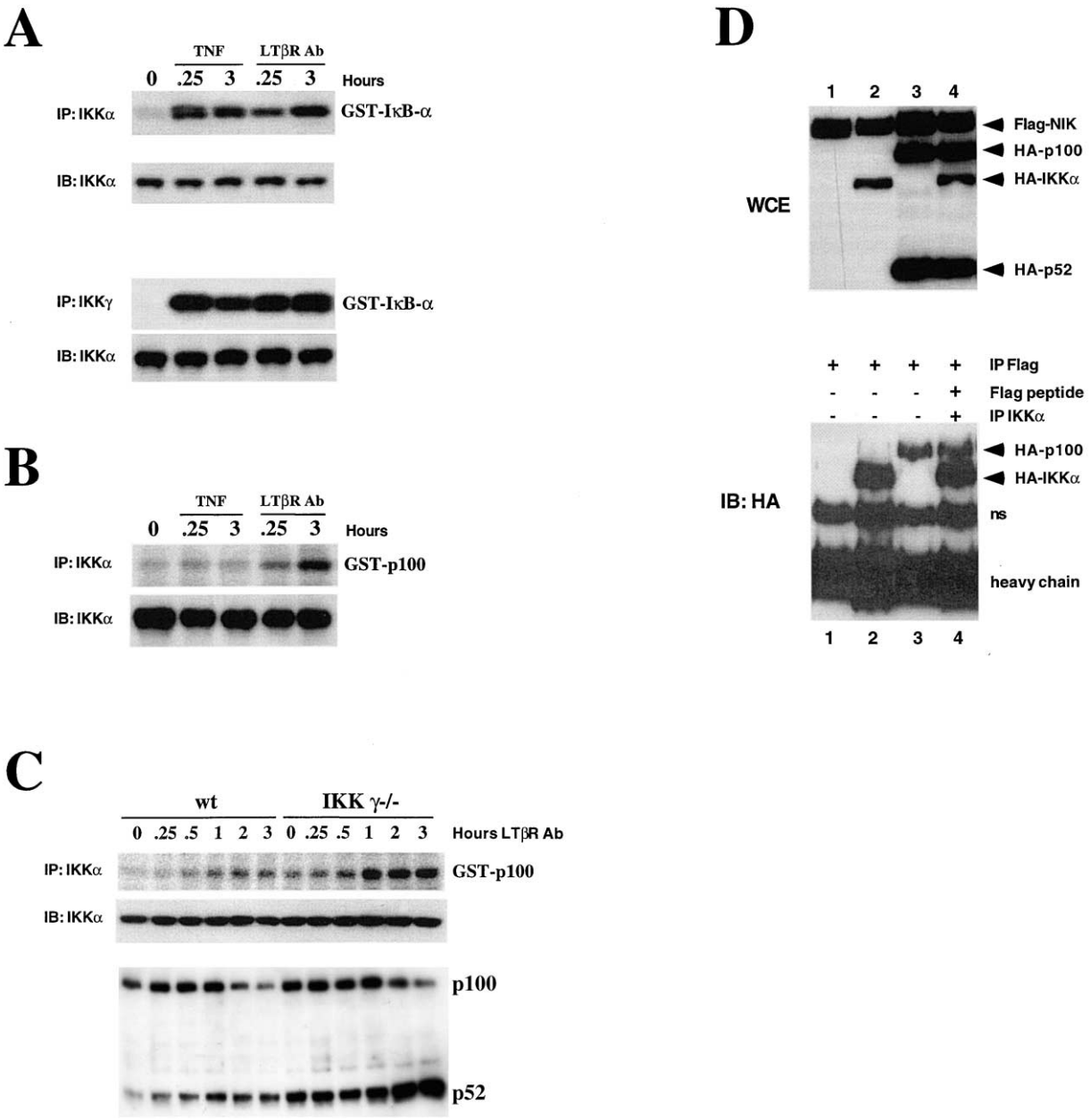

Figure 6. LTβR Ligation Induces p100 Processing through an IKKα-Containing Complex

(A) LTβR induces IκB-α kinase activity. Wild-type MEFs were treated for the indicated period of time with TNF (0.5 nM) or agonistic LTβR antibody (2 μg/ml). Cell extracts were subjected to immunoprecipitation with either anti-IKKα antibodies or anti-IKKγ antibody prior to kinase assays using a GST-IκB-α 1-54 substrate.

(B) LTβR but not TNFRI signals through IKKα to trigger p100 phosphorylation. Wild-type and IKKγ-deficient MEFs were stimulated as described in (A). Cell extracts were subjected to IKKα immunoprecipitation prior to kinase assays with a GST-p100 substrate.

(C) IKKγ is dispensable for LTβR-mediated p100 phosphorylation and processing. Wild-type and IKKγ-deficient embryonic fibroblasts were treated for the indicated period of time with 2 μg/ml of agonistic anti-LTβR. Cell extracts were immunoprecipitated with anti-IKKα antibodies and used for kinase assays with a GST-p100 substrate. The same extracts were analyzed by Immunoblotting to detect IKKα and p100:p52 protein expression.

(D) NIK, IKKα, and p100 can form a complex. 293T cells were transfected with constructs for expression of flag-NIK, HA-IKKa, and/or HA-p100, as indicated. Total expression of the proteins in whole cell extracts (WCE) is shown in the upper panel. In the lower panel, extracts were precipitated with anti-flag and tagged proteins detected by immunoblot with anti-HA antibody. In the lane on the far right, protein was precipitated with anti-flag, then eluted by incubation with flag peptide. The supernatants were then immunoprecipitated with anti-IKKα, and tagged proteins were detected by immunoblot with anti-HA antibody.

al., 1999; Li et al., 1999a; Takeda et al., 1999). Conversely, the processing of p100 is dependent on IKKα kinase activity but not on IKKβ (Senftleben et al., 2001). Using mice with a knockin mutation within the T loop domain of IKKα (Cao et al., 2001), we observed that IKKα activation is also required for LTβR-induced ex-

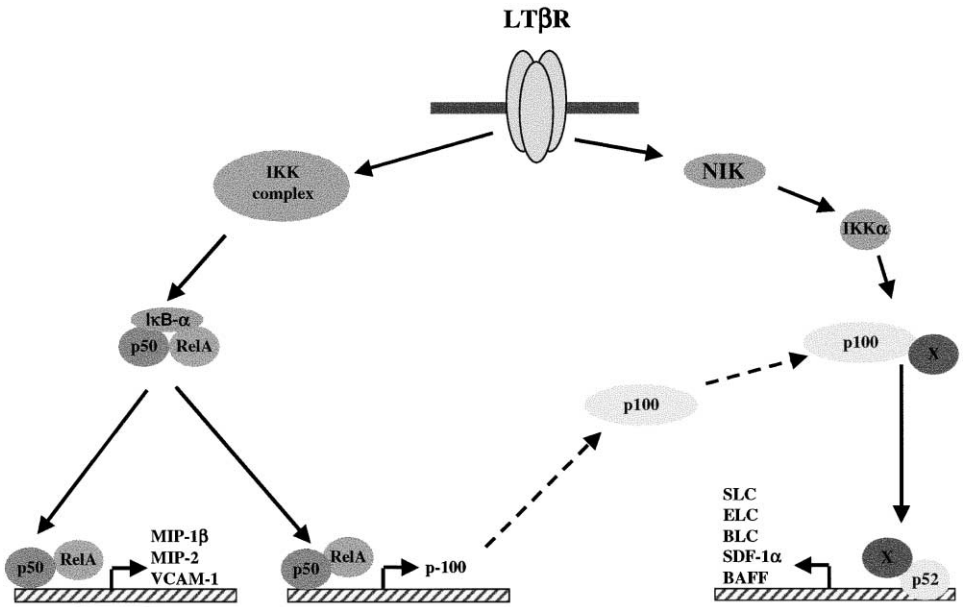

Figure 7. Model for Lymphotoxin-β Receptor-Mediated NF-κB Activation

Upon LTβR ligation, two NF-κB activating pathways are engaged. The first leads to activation of IKKβ and RelA, which control expression of inflammatory genes such as VCAM-1, MIP-1β, and MIP-2. In addition, this pathway leads to an increase of NF-κB2/p100 precursor. The processing of the latter is controlled by a second pathway that involves the activation of NIK which in turn activates IKKα for generating active p52. p52 in association with its partners (e.g., RelB) translocates to the nucleus and activates the transcription of genes implicated in secondary lymphoid organogenesis and homeostasis such as SLC, ELC, BLC, SDF-1α, and BAFF.

pression of the chemokines ELC, SLC, BLC, and SDF-1-α and the cytokine BAFF (Figure 2). SLC and ELC act via the CCR7 receptor and have been shown to have important functions in lymphoid homing to secondary lymphoid tissues (Forster et al., 1999; Gunn et al., 1999; Ngo et al., 2001; Reif et al., 2002). Similarly, it has been observed that expression of SLC and ELC, which play important roles in the organization of lymphoid organs, is reduced in p52 and RelB-deficient mice (Weih et al., 2001). SDF-1 plays roles in the early stages of B cell development (Egawa et al., 2001; Luther et al., 2000). In addition, BAFF has a critical role in B cell development, in the formation of germinal centers, and in B cell survival (Batten et al., 2000; Schiemann et al., 2001). The ability of LTβR to stimulate production of these mediators is consistent with its role in secondary lymphoid organogenesis and homeostasis.

It is therefore tempting to conclude that the NF-κB2 signaling pathway we have described links the LTβR receptor to critical chemokines and cytokines that account for the phenotypes of the knockouts discussed above. However, this is probably overly simplistic. IKKα plays important roles in signaling from receptors other than LTβR; for example, B cells (which lack LTβR) show IKKα-dependent p100 processing (Senftleben et al., 2001). Further, IKKα can function in the activation of conventional NF-κB as well (Cao et al., 2001). Several of the chemokines and cytokines we have examined may be produced in response to signals distinct from those of the LTβR -IKKα - NF-κB2 pathway we have discussed. For example, as in LTβR-deficient animals, we observed normal numbers of $CD3^+$ and $CD19^+$ cells in the spleens of 5-week- old knockin *Ikk*α^{AA} mice (data not shown). A defect in B cells observed in the latter animals appears around 9 weeks and becomes significant 12 weeks after bone marrow transfer (Senftleben et al., 2001), and may therefore be due to signaling defects that are distinct from those of the LTβR. In addition, and apart from these considerations, the cell types responsible for LTβR-ligation-dependent cytokine and chemokine production in the spleen have yet to be definitively determined, as this receptor has been identified on both stromal and myeloid cells (Browning and French, 2002).

Recent evidence has shown that although *tnfrl*$^{-/-}$ mice have minor defects in splenic architecture and Peyer's patch development (Neumann et al., 1996), *tnfrl*$^{-/-}$ *rela*$^{-/-}$ mice lack the same secondary lymphoid organs and structures that are absent in LTβR-deficient mice (Alcamo et al., 2001, 2002; Futterer et al., 1998). In contrast, animals lacking p50 do not show these developmental defects (Sha et al., 1995). Therefore, the RelA-p52 complex that we have observed following ligation of LTβR (Figure 5B) may be important in at least some of the IKKα-dependent gene expression required for organogenesis. However, the ability of RelA to induce p100 mRNA upregulation required for a sustained p52 production (Figures 2 and 3) may also play a significant role in LTβR-mediated lymphoid organogenesis.

It may also be that p52:RelB, induced by LTβR, tempers the inflammatory effects of LTβR-mediated signaling pathways, such as the canonical NF-κB activation pathway. We observed that the ability of ligated LTβR to induce the expression of the inflammatory chemokines MIP-1β and MIP-2 was enhanced in animals lacking inducible IKKα kinase activity (Figure 2). Interestingly,

RelB-deficient mice display a generalized inflammation (Burkly et al., 1995; Weih et al., 1996). Thus, p52:RelB, induced by LTβR, may function in the repression of these and other inflammatory chemokines. Therefore, the interplay of the different NF-κB pathways engaged by ligation of LTβR may be complex and contribute to inflammation and immune development in diverse ways.

Experimental Procedures

Plasmid Constructs and Retrovirus Gene Transfer

Wild-type human *Ikk*α and mutant *Ikk*α^{AA} (Delhase et al., 1999) were cloned into pMX-IRES-GFP retroviral vector. The following primers were used to PCR amplify human IKKα: S-5′-TATAGAATTCATG GAGCGGCCCCCGGGG-3′ and AS- 5′-TATAGTCGACTCATTCTGT TAACCAACTCC-3′. PCR products were digested with EcoRI and SalI and cloned into the retroviral vector digested with EcoRI-XhoI. Retroviral constructs in combination with the pEC-ampho expression vector were transfected by the calcium phosphate method into the 293T cell line. Two days later, the supernatants were collected and filtered prior to transduction of *Ikk*$\alpha^{-/-}$ MEFs. GFP positive cells were sorted to reach >95% GFP expressing cells. Expression vector for human p100 was used as template to PCR amplify the carboxy-terminal region (from amino acid 690 to 900) of p100 using the following primers: S-5′-TATAGGATTCGCTGGTGCTGACATCCATG CTG-3′, AS-5′-TATAGAATTCTCAGTGCACCTGAGGCTGGGGGT GCC-3′. The PCR product was digested by BamHI and EcoRI and cloned into pGex-4T1 (Pharmacia).

Generation of the LTβR Agonistic Antibodies

The anti-mouse LTβR monoclonal antibody (IgG1) was produced from a Sprague-Dawley rat immunized with mouse LTβR-Fc decoy protein (Force et al., 1995). This antibody is agonistic for the murine LTβR and does not ligate other TNFR family proteins (data not shown). Methods for endotoxin-free production and characterization have been described previously (VanArsdale and Ware, 1994). Mouse and goat anti-human LTβR has been described (Browning et al., 1995; Force et al., 2000).

Analysis of In Vivo Gene Expression

Five-week-old wt *Ikk*α mice and *Ikk*α^{AA} knockin mice (Cao et al., 2001) were injected intraperitoneally either with 50 μg of isotype control antibody (purified rat IgG1 K, 553921, Pharmingen) or with 50 μg of agonistic anti-LTβR monoclonal antibody. Eight hours later, splenocytes were isolated and RNA was reverse-transcribed using M-MLV reverse transcriptase and random hexamers (Gibco BRL). Real Time PCR was performed in a PE Biosystems 5700 thermocycler using SyBr Green detection protocol as outlined by the manufacturer. In brief, 12 ng of total cDNA, 50 nM of each primer, and 1X SyBr Green mix were used in a total volume of 25 μl. 18S RNA was used as reference. Primer sequences are available upon request.

Immunoblot, Coimmunoprecipitation, and Kinase Assays

Cell extracts were prepared as described previously (Dejardin et al., 1995) and analyzed by SDS-PAGE and immunoblotting with specific antibodies. For kinase assays, cells were lysed in a buffer containing Tris 20 mM (pH 7.6), NaF 20 mM, β-glycerolphosphate 20 mM, sodium orthovanadate 0.5 mM, EDTA 1 mM, EGTA 0.5 mM, glycerol 10%, CHAPS 0.5%, DTT 2 mM, PNPP 1 mM, NaCl 10 mM, and a cocktail of protease inhibitors. Samples were incubated on ice for 15 min and then centrifuged for 10 min at 14000 rpm. 50 μg of protein extract was immunoprecipitated overnight at 4C with a mix of two different anti-IKKα antibodies (M-280 and H-744, St-Cruz) or with anti-IKKγ antibody (Pharmingen) and protein A/G agarose beads. Kinase assay reactions and GST fusion protein production were as previously described (Delhase et al., 1999).

Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared and analyzed as previously described (Dejardin et al., 1999) using the probe: 5′AGTTGAGGGGAC TTTCCCAGGC3′.

Cell Culture and Cell Lines

Embryos from *LTβR*$^{-/-}$ (from Klaus Pfeffer, University of Munich, Munich, Germany) and Nik$^{aly/aly}$ (from CLEA Japan, Osaka, Japan) mice were used to prepare MEFs, expanded for two passages before doing an assay. IKKα, IKKβ, and IKKγ-deficient cells were described (Hu et al., 1999; Li et al., 1999c; Makris et al., 2000). MEF were cultured in DMEM (Gibco BRL) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. HT29 (ATCC) was cultured in McCoy's 5a with the same supplements.

Reagents and Antibodies

MG-132 and PNPP were from Calbiochem, zVAD-fmk was from Enzyme Systems Products. Recombinant human lymphotoxin α1β2 was from R&D Systems Inc.. Antibodies were from Imgenex (IKKα), Upstate Biotech. Inc. (p52:p100), Pharmingen (IKKγ), Santa-Cruz (RelA, RelB, p100/p52, NIK, IKKα), ICN (actin) and StressGen (Hsp60). The anti-p52:p100 polyclonal antibody was generously provided by J. Hiscott and N. Rice (Pepin et al., 1994).

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